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ANTI-ULCEROGENIC EFFECTS OF *NIGELLA SATIVA* L. MELANIN

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ABSTRACT

Aim: The current study is to examine the effect of a Herbal Melanin (HM), obtained from the seed coats of *Nigella sativa* L., as anti-ulcerogenic agent against alcohol-, indomethacin-, aspirin-, stress- and combined stress and aspirin-induced gastric ulcers in rats. **Method:** Using Wistar rats, gastric ulcers have been induced by administration of alcohol-, indomethacin-, aspirin-, stress and combined stress and aspirin. Herbal melanin was administrated before or together with the ulcerogenic agents. **Results:** Pretreatment or co-administration of HM with the ulcerogenic agents at doses of 0.12 or 1g kg^{-1} body weight, by gavage, significantly protected the stomach against inducible ulcers. **Conclusion:** Our findings suggest that HM, extracted from the seed coats of *Nigella sativa* L, strongly protects from ulcers induced by alcohol, indomethacin, stress, or the combined ulcerogenic action of both stress and aspirin.

KEYWORDS: Herbal melanin; Experimental ulcers; alcohol; indomethacin; aspirin; stress.

1. INTRODUCTION

Nigella sativa L. (Black cumin) has been used as a household herbal medicine and culinary ingredient in the Middle and Far East for millennia. The extensive reviews by Ali and Blunden[1] and Salem[2] show that the whole seed and its oil has been applied to a large number of ailments, including, asthma, diarrhoea, obesity, hypertension and gastro-intestinal diseases. Recently, it has been shown that the seed coats of *Nigella sativa* L. contain abundant amounts of herbal–melanin (HM).[3,4] Melanins are brown-black natural pigments that are widely produced by animals, plants and microorganisms[5] and have previously been

reported as anti-ulcerogens and anti-inflammatory agents.[6,7,8,9,10] All melanins comprise free radicals as a permanent fraction of their chemical structure. These stable free radicals may easily be detected by electron spin resonance (ESR) at room temperature.[11] In addition to this property, melanins also exhibit a strong antioxidant property that is related to their photo-protective and chemo-protective roles in living organisms.[12,3]

Melanin of *Nigella sativa* has been shown to induce cytokine production[4,13] and has been suggested as a ligand for Toll-like receptor 4 (TLR4).[13] TLRs are transmembrane receptors that share a high homology to the Toll receptors originally identified in *Drosophila melanogaster* and are, similarly, able to recognize conserved patterns on pathogens and microbes.[14] Various studies have demonstrated the expression of the TLR members in the mucosal layer of the gastrointestinal tract, including the stomach lumen.[15,16]

The mucosal layer protects the gastrointestinal tract from bacterial and microbial attacks where TLRs play a crucial role in the protection as has been reported by various studies.[17,18,19,20] Nevertheless, still the search for natural and synthetic drugs acting as prophylactics or treatments against gastric ulcers induced by stress, inflammation, bacteria, and other ulcerogenic agents remains an important pharmacological endeavor.

The anti-ulcerogenic effects of *Nigella sativa* L. melanin against induced ulcers, prior to this study, have not been investigated. We report here anti-ulcerogenic effects of herbal melanin on experimentally-induced ulcers in rats. We have found that HM, extracted from *Nigella sativa* L, strongly protects from ulcers induced by alcohol, indomethacin, stress, or the combined ulcerogenic action of both stress and aspirin.

2. MATERIALS AND METHODS

2.1. Preparation of HM

The extraction and preparation of highly pure melanin powder from the seed coats of the *Nigella sativa* L. plant have been carried via alkali solublization and acid aggregation as described before.[4] The melanin nature of the extract has been verified via the standard analytical techniques including ESR, infra red (IR), ultraviolet-visible (UV-VIS), Nuclear Magnetic Resonance (NMR), XRD, Fluorescence, Solubility studies, amino acid composition and elemental analysis.[4] The dry powder was stored and used later to prepare solutions at pH 7 for biological studies by re-dissolving the desired amount of melanin powder (in w/w ratio) in NaOH solutions (at pH 12.5) and using conc. HCl to adjust the pH to 7.

2.2. Experimental animals

Male Wistar rats (250 g body weight), provided by the Experimental Animals Care Centre, College of Pharmacy, King Saud University, Riyadh, have been used in this study. The animals were divided into groups (N = 6 animals per group). The distribution of animals in the different groups was randomized. The animals were maintained at 22 ± 1^{o}C on a 12h light dark cycle and given rat chow and water *ad libitum*. Food was withdrawn 48 hours before start of the experiments but tap water was allowed freely. All the experimental protocols were approved by the Animal Care Committee, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

2.3. The Ulcer Index

Following the method described by a number of authors.[21,22,23,24] a scoring system of Zero to Three, based on the number and the severity factor of the ulcer, has been employed**.** Accordingly, the severity factor of the ulcer was defined in relation to the length of the ulcer (lesion) as follows: Severity factor **Zero** = No visible lesions, Severity factor **One** = Lesion < 2 mm, Severity factor **Two** = Lesion 2 - 4 mm and Severity factor **Three** = Lesion > 4 mm. The ulcer index was calculated as the total number of ulcers per stomach multiplied by the severity factor. To asses the ulcer index subsequent to each experiment the abdomen of each animal was opened, the stomach was removed and cut along the greater curvature and gently rinsed with isotonic saline. The stomach was then pinned out on a flat surface with the mucosal surface uppermost. The levels of severity of the induced gastric ulcers were assessed by calculating the Ulcer Index as described above.

2.4. Induction of ulcers by alcohol

Gastric ulcers were induced via administration of 1 ml 80% ethanol per rat by gavage. Herbal melanin, in selected doses of 0.12 g and 1 g kg^{-1}, was administered to the test group one hour before the administration of alcohol in at dose of 8 ml kg^{-1}, by gavage. One hour after administration of alcohol all rats were sacrificed by cervical dislocation and the ulcer index was calculated as described above.

2.5. Induction of ulcers by indomethacin

To induce ulcers by indomethacin the drug was suspended in 0.25% sodium carboxy methyl cellulose in water and homogenized thoroughly. Ulcers were induced in the control group by administration of 60 mg kg^{-1} of indomethacin, using a modification of the method described by previous authors[24,25] in doses of 4 ml kg^{-1} by gavage. One hour before indomethacin

administration rats in one test group were administered HM by gavage in doses of 250 mg kg^{-1}, in water volumes of 4 ml kg^{-1}. Rats in another test group were administered HM together with indomethacin in a similar manner. All animals were killed six hours following administration of indomethacin, the stomachs were removed and the ulcer index was calculated as described above.

2.6. Induction of ulcers by stress

Stress–induced ulcers were obtained in the rats by a modification of the method described by Senay and Levine.[26] and Levine.[27] The 48-hour food deprived rats were immobilized in restraint cages and placed inside a ventilated refrigerator maintained at a temperature of 4^{o}C. The animals were killed and their stomachs examined after four hours of stress treatment. To test the influence of HM on the induced ulcers each rat was given HM in a dose of 0.12 g kg^{-1} contained in a water solution volume equivalent of 8 ml kg^{-1} one hour before exposure to stress.

2.7. Induction of ulcers by stress and aspirin

Ulcers were induced in rats by the combined effects of stress and aspirin.[28,29] Aspirin was suspended in 0.25% sodium carboxymethyl cellulose and homogenized as described above for indomethacin. Each rat in the control group received an aspirin dose of 100 mg kg^{-1} by gavage in a volume equivalent to 4 ml kg^{-1} and immediately exposed to stress for 4 hours as described above. To test the effect of HM on the induced ulcers, HM was administered in a dose of 0.12 g kg^{-1} in a volume equivalent to 8 ml kg^{-1} by gavage together with aspirin. After the 4 hours of cold-exposure time, all rats were killed by cervical dislocation, their stomachs removed and the ulcer index calculated as described above.

2.8. Effect of Ranitidine

For comparative assessment, ranitidine -a standard anti-ulcerogenic drug[30] was orally administered in doses of 0.12 g kg^{-1} one hour before the exposure of the animals to the different ulcerogens.

2.9. Statistical analysis

Statistical means were evaluated in order to determine the ulcer indices. All data were expressed as means with standard errors. Significance differences between the groups were made by the use of the student's "t" test for unpaired data (when a single dose of melanin was used) and by analysis of variance (when two doses of melanin were used). A ***p*** value of <

0.05 was considered significant. Table 1 (a, b, c, d) shows the cumulative results of the mean values of ulcer indices ± standard errors for all experiments.

3. RESULTS

3.1. Effect of HM on alcohol-induced ulcer

Administration of 80% aqueous alcohol to rats orally resulted in excessive hemorrhagic ulcers as shown in Fig. 1.a. Pre-treatment of the animals with herbal melanin in doses of 0.12 and 1 g kg^{-1} suppressed the ulcer formation. The experimentally obtained ulcer indices were 19.64 ± 2.31 for control experiments, 11.66 ± 1.72 ($p < 0.05$, N = 6) for 0.12 g kg^{-1} HM doses and 1.66 ± 1.25 for 1g kg^{-1} HM doses ($p < 0.001$, N = 6). As may be seen from Table 1.a., administration of alcohol together with the larger dose of HM (1 g kg^{-1}) significantly suppressed the induction of ulcers as shown in Fig. 1.b.

3.2. Effect of HM on indomethacin-induced ulcers

Administration of oral indomethacin to rats induced a larger number of small gastric ulcers as shown in Table 1-b, Fig. 1.c. However, the induced ulcers were smaller and the hemorrhage was far less severe when compared to alcohol. Pre-treatment of rats with HM in a dose of 250 mg kg^{-1} or co-administration of the same dose with indomethacin significantly suppressed ulcer indices from 51.82 ± 8.06 to 12.83 ± 4.99 ($p < 0.01$, N = 6) and 6.38 ± 4.25 ($p < 0.001$, N= 6) (Fig. 1.c and 1-d).

3.3. Effect of HM on stress induced ulcer

Exposure of the rats to hypothermia-induced stress resulted in appearance of ulcers that were effectively prevented by pretreatment of the rats with HM in oral doses of 1 g kg^{-1}. As shown in Table 1-c the means of the stress-induced ulcer indices for the control group and the HM treated rats were 14.16 ± 4.11 and 0.83±0.52 ($p < 0.02$, N = 6), respectively.

3.4. Effect of melanin on ulcers induced by stress+aspirin

Response of rats to exposure to the combined influence of aspirin (100 mg kg^{-1} orally) and hypothermic stress induced gastric ulcers is as shown in Table 1-d and Fig. 1.e. Ulcers production was enhanced 2 to 3 times when compared to those observed in the case of stress alone. The ulcer index was 37.83 ± 6.8 in the case of the “aspirin+stress” treated rats and was reduced to 2.66 ± 0.57 ($p < 0.001$, N= 6) in the HM treated groupas shown in Fig. 1.f.

3.5. Effect of Ranitidine (standard drug) on chemicals and stress-induced ulcers

Table 2 shows results of treatment of rats with ranitidine, 120 mg/kg orally, one hour before exposure of the animals to the above ulcerogens. The ranitidine treated animals showed ulcerogenic indices of 10.1±1.3, 20.7±1.9, 8.3± 0.4 and 14.7 ± 1.1 following the administration of ulcerogens alcohol, indomethacin, stress and the combined "aspirin and stress", respectively ($P < 0.05$, $N = 6$).

Table 1

Table 1-a. Effect of melanin on alcohol induced ulcers.

Treatment group and dose	*Ulcer Index*
Control	19.64 ± 2.31
Melanin 0.12 g kg^{-1}	11.66 ± 1.72*
Melanin 1 g kg^{-1}	1.66 ± 1.25 **

**p <0.05, N = 6; compared with control.*

***p <0.001, N = 6; compared with control.*

Table 1-b. Effect of melanin on indomethacin induced ulcers.

Treatment group and dose	*Ulcer Index*
Control	51.82 ± 8.06
Melanin 250 mg kg^{-1} pre-treatment	12.83 ± 4.99*
Melanin 250 mg kg^{-1} co-administration	6.38 ± 4.25**

**p < 0.01, N = 6 compared with control.*

**p < 0.001, N = 6 compared with control.*

Table 1-c. Effect of melanin on stress-induced ulcers.

Treatment group and dose	*Ulcer Index*
Control	14.16 ± 4.11
Melanin 0.12 g kg^{-1}	0.83 ± 0.52*

**p <0.02, N = 6; compared with control.*

Table 1-d. Effect of melanin on "stress and aspirin"-induced ulcers.

Treatment group and dose	*Ulcer Index*
Control: stress+aspirin	37.83 ± 6.8
Melanin 0.12 g kg^{-1}	2.66 ± 0.57*

**p <0.001, N = 6; compared with control.*

Table 2

Table 2. Effect of Ranitidine on chemicals and drug induced ulcers.

Treatment group and dose	*Ulcer Index*
Alcohol 0.12 g kg^{-1}	10.11 ± 1.32*
Indomethacin 0.12 g kg^{-1}	20.73 ± 1.95*
Stress 0.12 g kg^{-1}	8.32 ± 0.48 *
Stress +aspirin 0.12 g kg^{-1}	14.77 ± 1.12*

**p <0.05, N = 6.*

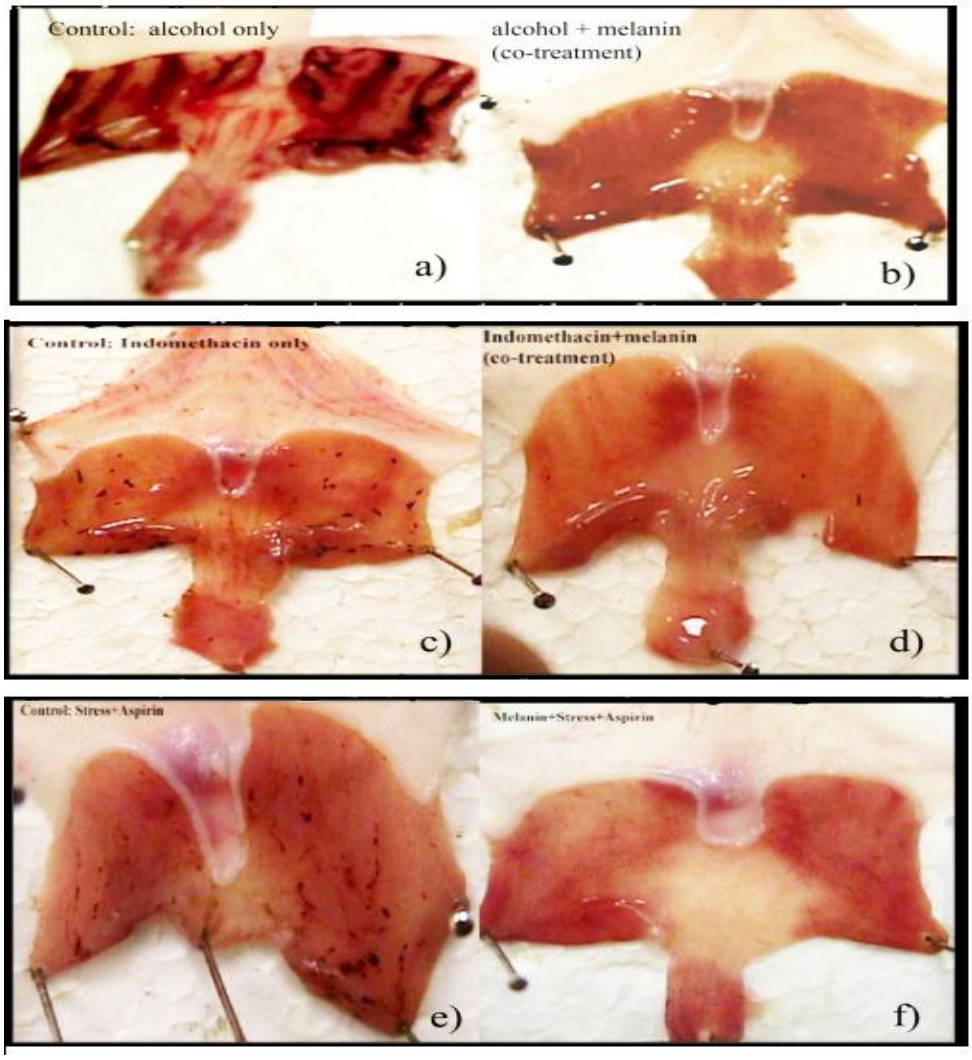

Figure 1. Ulcer inducing agents(alcohol, indomethacin and stress+asprin) were administered to the rats either separately (a, c, e) or after administration of melanin (b, d, f).

4. DISCUSSION

The results, above, indicate that herbal melanin (HM) extracted from *Nigella sativa* L. has a strong anti-ulcerogenic effect on Wistar rats. The HM used was effective whether

administered before or co-administered with the ulcerogenic agent. It provides a significant anti- ulcerogenic action against alcohol-, indomethacin-, aspirin-, stress- and combined stress and aspirin-induced gastric ulcers. Herbal melanin protective activity was greater than that of Rantidine the well known standard anti-ulcer drug.

Previously, we have shown that HM induces cytokines production and suggested a role for HM as a TLR4 ligand.[4,13] We have also pointed out the similarity between HM and LPS as TLR4 ligands.[13] Recently, many studies have shown a protective role of different TLR4 ligands against induced gastrointestinal injuries.[17,18,19,20] Medzhitov *et al.* (2004).[20] demonstrated that commensals colonizing in the stomach protect the stomach from injuries by activating TLR4. In one of their experiments they eliminate the stomach bacteria by administering antibiotics to mice and subsequently giving them the TLR4 ligand, LPS, together with the ulcer inducing agent dextran sulfate sodium (DSS). They found that LPS has completely protected the rats from DSS-induced ulcers. They concluded that protection is obtained by the activation of TLR 4 by LPS and by induction of cytokines[20]

Fukata, *et al*. 2006[17] have reported that LPS/TLR4 activation can also protects the gut by inducing cyclo-oxygenase (COX)-2 and prostaglandin (PG)E2. They showed that TLR4 signaling induces COX-2 expression in lamina propria macrophages and in the epithelial cells, which results in up-regulation of mucosal PGE2. They proposed that this up-regulation of PGE2 might be required for mucosal restitution in response to epithelial injury. Moreover, Hoogerwerf *et al*. (2006)[31] reported that a disruption in the synthesis of cytoprotective prostaglandins (PGE2 and PGI2) by inhibition of the constitutive form of cyclooxygenase (COX- 1) in the mucous is considered one of the main mechanisms of the formation of peptic ulcers and duodenal disorders. Zheng *et al*. (2009) reported hyaluronic acid (HA) as another TLR4 ligand for the protection against DSS-induced colitis and they showed that intraperitoneal, endogenous and exogenous administration of HA had activated TLR4, induced COX2 and subsequently prostaglandin E2.[18] Later on, Chen H. *et al.* 2011.[19] showed that a high molecular weight hyaluronic acid, HMWHA, has protected from induced-gastrointestinal colitis via activation of TLR4 and expression of Cox-2 and PGE2 expression. The similarity in effects between HM and TLR4 ligands suggests similar protective action of HM in the gastrointestinal tract via HM/TLR4 activation and Cox-2 mediated production of PGE2.

Other possible means for protection of the mucosal layer from injuries via TLR4 was suggested to be mediated by the secretion of anti-microbial peptides such as Beta-defensin-2.[32] Biragyn *et al.*[32] suggested that Beta-defensin-2 acts as a ligand for TLR4[33], whereas Ohara *et al.* suggested that Beta-defensin-2 can act as a pro-inflammatory mediator that transmits signals from the mucosal surface to the sites of gastric mucosal damage.[34] The related issues of whether the protective effects of HM against induced ulcers are attributed to TLR activation by HM or by defensin-2 production merit further detailed studies.

Ligand activation of TLR4 induces cytokines production via the NF-kB signaling pathway.[35,36] The increased NF-κB activation has been considered as a pathogenic factor that affects many inflammatory disorders.[37,38] Recently many studies have shown that NF-κB inhibition can also be harmful. Wullart *et al.* 2010[39], have reported the role of NF-Kb signaling pathway in protection against gastrointestinal injuries. Similarly, Takahashi *et al.* (2001).[40] studied the role of NF-kb in gastric ulcer healing in rats and demonstrated that the persistent inhibition of NF-kB in ulcerated tissues has caused an impairment of ulcer healing. Recently, Shibata *et al.* 2010[41] showed that deactivation of NF-kB signaling in gastric epithelial cells resulted in increased necrosis and more rapid progression to gastric preneoplasia. Previously, it has been shown that HM has activated the TLR4/NF-kB signaling pathway**.**[42]

Early pharmacological investigations regarding the biochemical causes of alcohol-induced ulcers implicated oxygen-derived free radicals[43] and a decrease in the mucosal level of non-protein sulfhydryl compounds as a cause of the ulceration.[44] Indomethacin and aspirin-induced ulcers are believed to be mediated by an inhibition of gastric prostaglandins (derived via the prostaglandins endoperoxide synthase COX-1-enzyme).[28,45,46] leading to increased free radical formation.[47] Stress induced ulcers are thought to involve an increase in oxygen free radicals[29], an increase in gastric acid secretion[48], a reduction in gastric glutathione content[49] and a decrease in gastric PGE2 production.[50] In all these models the common mechanism is an increase of the production of reactive oxygen free radical species (ROS). Since melanin has also been shown to suppress production of free radicals.[7,8] it is pertinent to suggest that the anti-ulcerogenic effects of melanins could be related to this property. It is also interesting to note that the authors, originally reporting on the anti-ulcerogenic activity of marine-animal melanins (on pylorus-ligated, phenylbutazone- and aspirin-induced ulcers[6,7,8],

suggested that melanin protects from ulcers by increasing the biosynthesis of glycoproteins, or by decreasing their degradation, in the gastric mucosa.[7]

Our previously reported electron spin resonance (ESR) studies[3,4] have revealed a substantial occurrence of melanin in the seed coats of this well known and widely used medicinal and culinary herb. Melanin represents around 15% of the seed coat alone; amounting to around 2.5% of the total mass of the *Nigella sativa* L. seed. Melanin pigments are well known for their various versatile biophysical and biochemical roles.[5] Some authors have demonstrated anti-ulcerogenic effects for *Nigella sativa* L total extracts or its constituents such as the volatile oil component: thymoquinone as well as its fixed seed oil.[51,52,53] However, those studies did not take note of the rich presence of melanin in this plant. This study is the first to demonstrate a protective effect of a herbal melanin extracted from *Nigella sativa* L. against ulcers.

5. CONCLUSION

In this study we have shown that HM significantly reduces alcohol, aspirin, stress and indomethacin-induced gastric ulcers. We, therefore, hypothesized that the HM has a possible role in the observed protection from ulcers by acting as a ligand for TLR4 and activating NF-kB signaling pathway. We suggest the use of herbal-melanin from *Nigella sativa* L. in future approaches for prevention and treatment of ulcers.

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